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RT-PCR Based Diagnosis and Molecular Characterisation of Mumps Viruses Derived From Clinical Specimens Collected During the 1996 Mumps Outbreak in Portugal

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Clinical specimens collected during an outbreak of mumps were characterised by RT-PCR, nested PCR, and nucleotide sequencing. Mumps virus was positively identified in 12/21(57%) saliva, 9/21(43%), throat and 1/33(3%) urine specimens and further sequence comparison revealed that at least six strains of viruses, which differed from 0–9.43% at the nucleotide levels, were co-circulating during the epidemic. However, phylogenetic analysis showed that these viruses grouped with two previously identified lineages which were mostly composed of other European mumps virus isolates. ***J. Med. Virol. 52:349–353, 1997.***

KEY WORDS: mumps virus; molecular characterisation; SH gene; genetic variation

INTRODUCTION

Mumps is a common childhood disease which causes symptomatic parotitis in most cases. About half of mumps infections develop into meningitis. In addition to infection of the parotid glands and the CNS, mumps virus can also cause orchitis, pancreatitis, oopharitis, mastitis, thyroditis, mycorditis, nephritis, arthritis, and deafness [for review see Leinikki, 1995].

In Portugal, as in other Western European countries, live attenuated mumps virus vaccine is given together with measles and rubella vaccine to children of 12–15 months of age. Until October 1992, the Urabe strain of mumps vaccine was used for immunisation. It was then replaced by a vaccine containing the Rubini strain and produced by a different manufacturer. The Urabe strain of mumps vaccine has been found to be associated with a high incidence of vaccine related adverse effects [Colville and Pugh, 1992].

Mumps virus is monotypic in nature, therefore, in theory, any immunogenic vaccine of a given strain should provide life long protection against subsequent infection. However, sporadic mumps outbreaks are reported even in countries which are known to have an excellent record of vaccine coverage [Briss et al., 1994; Künkel et al., 1994; Ströhle et al., 1996; Germann et al., 1996; Dias et al., 1996]. In order to distinguish whether the recent outbreak in Portugal was caused by endemic European strains or by imported genotypes, clinical specimens derived from patients were characterised by RT-PCR, nested PCR, and nucleotide sequencing. The genomic regions encoding the Small Hydrophobic (SH) protein gene of 12 isolates were sequenced and compared as this region has been found to be highly variable between strains and a large number of sequences are already available [Afzal et al., 1997]. The results show that multiple virus strains were co-circulating during the epidemic. However, the viruses share extensive sequence homology with other European isolates. This is the first report which describes the nucleotide sequence data of the SH genes generated by RT-PCR directly from salivary and other clinical specimens rather than viruses after isolation in culture.

MATERIALS AND METHODS

The clinical and vaccination history of all 21 subjects from which the specimens were collected is provided in Table I.

Clinical Specimens

Specimens of saliva, throat swabs, and urine were collected from clinically confirmed patients between

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TABLE I. The Clinical and Vaccination Details of Donors*

S. no.	Case no.	Sex	Age	Symptoms	Disease onset	Vaccination history	First collect	Second collect
1	Po1	F	5	P	n.a.	1992, MMR(Merieux)	16-05-1996	n.a.
2	Po3	M	8	P	13-05-1996	1990, MMR(Pluserix)	15-05-1996	31-05-1996
3	Po4	M	3	P	n.a.	1993, MMR(Berna)	15-05-1996	n.a.
4	Po6	M	8	P	08-05-1996	1989, 59A41C	15.05-1996	03-06-1996
5	Po7	M	4	P	01-05-1996	1993, MMR(Berna)	15-05-1996	31-05-1996
6	Po10	F	3	P	02-05-1996	1994, MMR(Berna)	15-05-1996	03-06-1996
7	Po14	M	5	P	n.a.	1990, MMR -	15-05-1996	n.a.
8	Po15	F	5	P, Erythema/ papulas	12-05-1996	1993, MMR(Berna)	15-05-1996	31-05-1996
9	Po17	F	3	P	n.a.	1994, MMR(Berna)	15-05-1996	n.a.
10	Po19	M	3	P	n.a.	?	15-05-1996	n.a.
11	Po20	F	10	P	11-05-1996	1988, TRSSC	15-05-1996	31-05-1996
12	Po21	F	7	P	n.a.	?MMR(Merieux)	16-05-1996	n.a.
13	Po22	M	10	P	n.a.	1994, MMR(Berna)	16-05-1996	n.a.
14	Po23	F	11	P	n.a.	?	16-05-1996	n.a.
15	Po24	F	4	P, Generalized Puringo	07-05-1996	1994, MMR(Berna)	16-05-1996	31-05-1996
16	Po26	M	5	P	14-05-1996	1993, MMR(Berna)	16-05-1996	31-05-1996
17	Po27	M	11	P	13-05-1996	1989, MMR(Pluserix)	16-05-1996	31-05-1996
18	Po30	M	8	P	23-04-1996	1989, MMR(Berna)	16-05-1996	31-05-1996
19	Po31	F	14	P	11-05-1996	1993, MMR(Berna)	16-05-1996	31-05-1996
20	Po32	M	42	P	19-04-1996	Unvaccinated	16-05-1996	n.a.
21	Po40	F	4	P, Erythema	10-05-1996	1994, MMR(Berna)	16-05-1996	31-05-96

*First collect includes saliva, throat swab, urine, and blood samples. Second collect includes urine and blood samples only. Po1, Portugal1; P, parotitis; n.a., not available.

15–16 May 1996 (Table I). All specimens except urine samples were collected with sterile swabs (Sterilin, Copan Italia) in the morning. The swab was then immersed immediately in 0.5 ml of viral transport media (Eagle's minimum essential medium [MEM] containing 1% BSA) and kept frozen on dry ice until transported to NIBSC, UK. The urine drops were collected without additions in sterilised tubes and stored at 4°C. In addition, where possible, blood samples were also collected from patients. Second urine and serum samples were collected from available subjects after 2 weeks (Table I).

RNA Extraction, RT-PCR, and Nucleotide Sequencing

For each clinical sample studied, a 250 μl aliquot was digested with proteinase-K, RNA was extracted with phenol-chloroform and chloroform and precipitated with ethanol [Sambrook et al., 1989]. Reverse transcription of RNA was carried out with random primers (dN6) as described previously [Afzal et al., 1997] and converted DNA was amplified with Taq polymerase in the presence of a set of primers (P1/P2) for an initial round of 30 cycles (94°C—1.0 min; 37°C—0.5 min; and 72°C—1.5 min). About 2.0 μl of RT-PCR products were re-amplified by nested PCR in the presence of a second set of internal primers (4889/4890) for another round of 30 cycles (94°C—1.0 min; 50°C—0.5 min; 72°C—1.0 min). The DNA products of RT-PCR and nested PCR amplifications were examined on agarose gels and, if required, by Southern blotting. The primers, purification of DNA products and nucleotide sequencing were as described [Afzal et al., 1993, 1994, 1997].

Virus Neutralization Assay

Two fold dilutions (1:4 → 1:128) of serum were prepared in Eagle's MEM containing 4% FCS (fetal calf serum) to a final volume of 50 μl and mixed with an equal volume of mumps virus tissue culture harvest containing approximately 30–40 p.f.u. The mixture was incubated for 1 hr at room temperature after which 1 ml of trypsinized Vero cell suspension was added and the mixture cultured in a 24 well tissue culture plate. After an initial incubation of 3 hr at 35°C the medium was replaced with fresh MEM containing 4% FCS and 0.8% carboxymethyl cellulose. The plate was incubated for a further 7 days at 35°C in a humidified CO_2 incubator and then stained with 0.5% methyl violet. The plaques were counted manually and data were analysed to determine neutralization end point 50 (NEP50). In this study serum samples were challenged with a tissue culture adapted wild type strain, Lo1/UK/88 [Afzal et al., 1997].

RESULTS

A Southern blot of primary RT-PCR DNA products of 21 saliva samples is shown in Figure 1. These products were not visible on the agarose gel stained with ethidium bromide. Analysis of the autoradiograph (Fig. 1) shows that the mumps probe derived from a cDNA clone (λMuS304) of the SH gene [Elliott et al., 1989] hybridised to DNA products in lanes 2, 5, 6, 7, 8, 9, 10, 16, 17, 19, 21 and very weakly to the product in lane 4

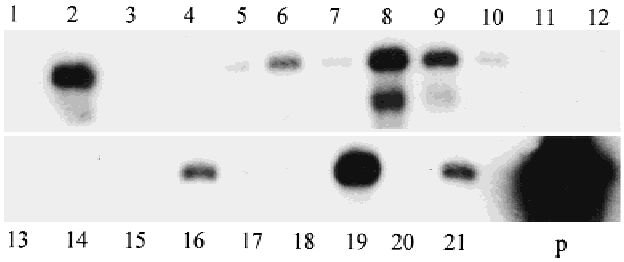

Fig. 1. Photograph of the Southern blot of primary RT-PCR products of 21 saliva samples (see Table I & II for details). P-mumps virus positive control.

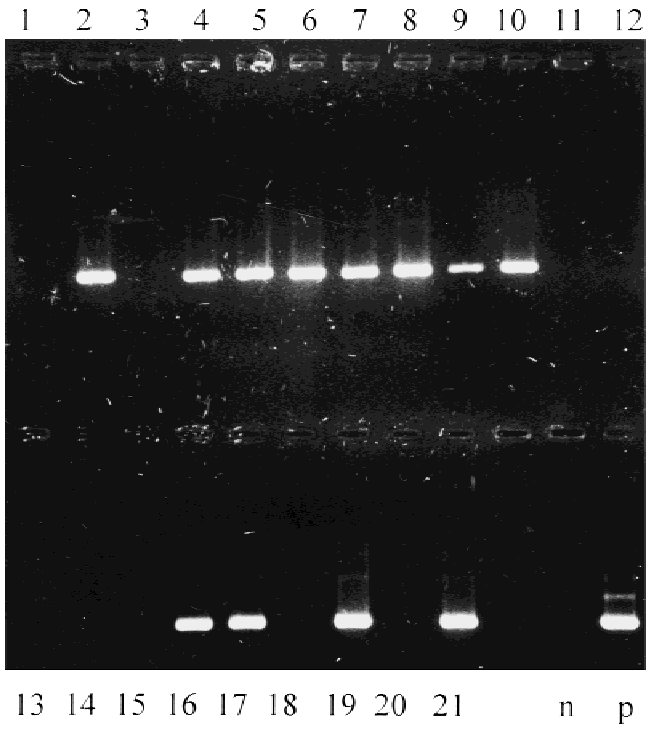

Fig. 2. Photograph of an agarose gel stained with ethidium bromide containing nested PCR DNA products of 21 RT-PCR samples shown in Figure 1.

which was visible following prolonged X-ray exposure (data not shown). About 2 μl of RT-PCR products were re-amplified by nested PCR and the resultant products examined by agarose gel analysis (Fig. 2). Comparison of the two figures shows that the positive samples identified on the blot (Fig. 1) were also positive in the nested PCR amplification (Fig. 2). This confirms the specificity of the blot and provides evidence for the lack of contamination during nested PCR amplification. Similar RT-PCR and nested PCR amplifications were also carried out with throat swabs and urine specimens of the patients and DNA products were examined by agarose gel analysis (data not shown). Altogether, mumps virus specific DNA fragments were generated from 12/21 (57%) saliva, 9/21 (42.86%) throat, and 1/33 (3%) urine specimens. The findings are summarised in Table II. The limits of detection were shown to be approximately 1 p.f.u. per reaction (data not shown).

Twelve of the 21 cases examined excreted mumps virus in saliva and of these twelve cases, nine excreted virus both in saliva and throat. Only one case (Po40) excreted virus in all three clinical specimens. All second urine samples examined were negative and this includes Po40 whose initial urine sample (collected at the 6th day post-infection) was positive. Fifty-eight percent (7/12) of the positive samples were collected within 10 days of the disease onset and 75% (9/12) within 14 days. The time of collection of samples from the three remaining positive cases (Po14, Po17, and Po19) was not known (Table I).

All sera examined except the serum derived from Po27 had significant levels of neutralization antibodies against mumps virus (Table II). From the first bleed serum samples derived from Po30 and Po32 showed the highest neutralization antibody titres, 1:>128 and 1:63, respectively while case Po27 showed the lowest level (<4). Analysis of the second bleed showed that serum antibody levels of all sera, except Po30, had increased significantly, consistent with recent mumps infection. It was not possible to assess IgM levels because there was insufficient serum.

The complete nucleotide sequences of the SH genes amplified from the 12 saliva and nine throat swabs were generated and compared. All isolates were labelled according to the new nomenclature [Afzal et al., 1997]. Of 12 sequences generated from saliva, Po7s/Portugal/96 and Po27s/Portugal/96 were identical to Po6s/Portugal/96. Similarly, Po14s/Portugal/96, Po15s/Portugal/96, Po19s/Portugal/96, and Po31s/Portugal/96 were identical to Po3s/Portugal/96. The remaining four sequences Po10s/Portugal/96, Po17s/

TABLE II. Summary of the Findings of Nested PCRs and Serum Neutrilisation Antibody Titre

S. no.	Case no.	Nested PCR results S	T	U^{1st}	U^{2nd}	NEP50 value first bleed Lo1/UK/88	NEP50 value second bleed Lo1/UK/88
1	Po1	−	−	−	n.a.	n.a.	n.a.
2	Po3	+	+	−	−	58	>128
3	Po4	−	−	−	n.a.	n.a.	n.a.
4	Po6	+	−	−	−	51	107
5	Po7	+	−	−	−	30	n.a.
6	Po10	+	+	−	−	24	>128
7	Po14	+	+	−	n.a.	53	n.a.
8	Po15	+	+	−	−	8	92
9	Po17	+	+	−	n.a.	n.a.	n.a.
10	Po19	+	+	−	n.a.	16	n.a.
11	Po20	−	−	−	−	17	99
12	Po21	−	−	−	n.a.	34	n.a.
13	Po22	−	−	−	n.a.	n.a.	n.a.
14	Po23	−	−	−	n.a.	18	n.a.
15	Po24	−	−	−	−	n.a.	31
16	Po26	+	+	−	−	23	42
17	Po27	+	−	−	−	<4	16
18	Po30	−	−	−	−	>128	65
19	Po31	+	+	−	−	28	>128
20	Po32	−	−	−	n.a.	63	n.a.
21	Po40	+	+	+	−	n.a.	24

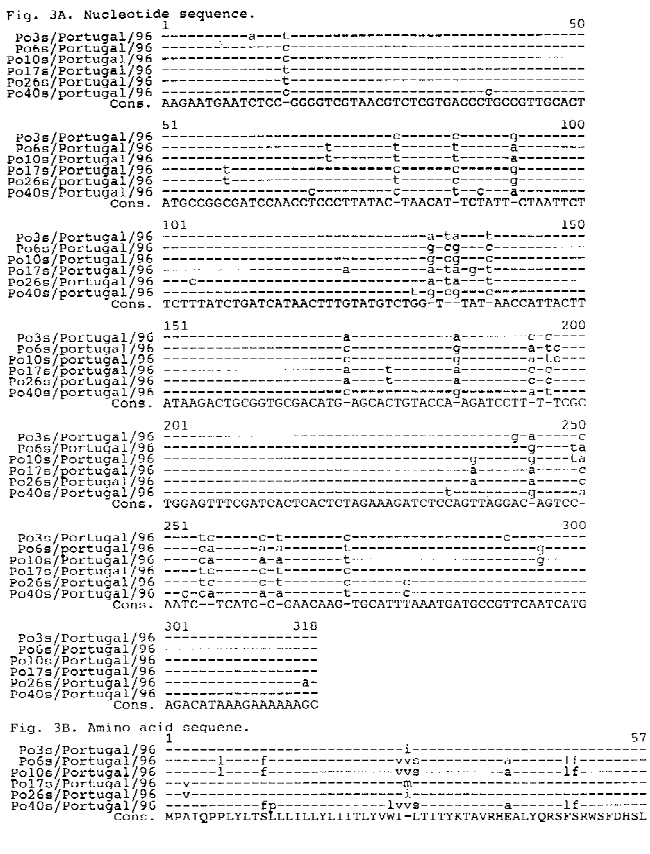

Fig. 3. Comparison of **(A)** nucleotide and **(B)** deduced amino acid sequences of the SH protein genes of six distinct viruses derived from the saliva samples.

Portugal/96, Po26s/Portugal/96, and Po40s/Portugal/96 were distinct. The six unique sequences identified have been lodged with the EMBL nucleotide sequence database under accession numbers Y08212-Y08217 and comparison of their nucleotide and deduced amino acid sequences is shown in Figure 3A and B, respectively.

The sequences generated from saliva and throat specimens of the same patients were identical in the SH gene region. The urine sample has not yet been sequenced and will be the subject of future study. The sequence of Po10s/Portugal/96 differed by only one nucleotide (position 237) from that of Po6s/Portugal/96, Po7s/Portugal/96, and Po27s/Portugal/96. This mutation is located outside the coding region of the SH gene and therefore the deduced amino acid sequences of Po6s/Portugal/96, Po7s/Portugal/96, Po10s/Portugal/96, and Po27s/Portugal/96 are identical. Strain Po40s/Portugal/96 differed by 30 nts (9.43%) and 10 a.a (17.24%) from both Po17s/Portugal/96 and Po26s/Portugal/96. However, the sequence of Po17s/Portugal/96 differed at six positions (1.89%) at the nucleotide level and by one amino acid from Po26s/Portugal/96. The open reading frame (ORF) of the SH protein gene, which starts at position 51 (AUG) and terminates at position 224 (UAG), was conserved in sequences from all clinical specimens. The new isolates were incorporated into an updated phylogenetic tree of the SH gene sequences (Fig. 4) essentially as described previously [Afzal et al., 1997].

DISCUSSION

This report describes the identification of mumps virus genome by RT-PCR and nested PCR amplification from unpassaged clinical specimens collected during the recent mumps outbreak in Portugal. While all specimens examined were collected from clinically symptomatic cases, the virus detection rates were significantly higher from saliva (57%) and throat (43%) than urine (3%). It is conceivable that cross-contamination from saliva to throat may have occurred *in vivo* as none of the throat swabs was positive where a corresponding saliva specimen was negative. In addition, viruses from both sources produced identical sequences in all cases examined. The low virus identification rate from urine suggests poor excretion or preservation of mumps virus in urine. However, we suspect that the severity of the disease probably governs how widespread the virus is *in vivo.*

Nine saliva, 12 throat, and 32 urine specimens remained negative, despite the fact these specimens were collected from clinically and serologically confirmed cases. Identification was more successful from clinical specimens collected during the early convalescent phase (within 10 days of post-infection) than from the specimens collected later.

At least six of the 12 virus sequences detected are distinct. We have identified three main lineage groups (A–C) with multiple subgroups composed of genetically similar viruses based on SH genes sequences [Afzal et al., 1997]. Seven of the 12 epidemic viruses belong to genotype group B, in subgroup B6, which is otherwise composed exclusively of viruses isolated in France and Germany. The remaining five belong to genotype group C in subgroups C5 and C7. None of the new sequences had significant genetic homology to the vaccine strains or non-European isolates.

Due to the limited number of cases analysed during this study, it is difficult to establish epidemiological links between individual patients. Substantial numbers of cases occurred in children of 1 to 4 years of age in kindergartens, schools, and other child care facilities and at least 40–60% of these had a history of mumps vaccination [Dias et al., 1996].

Co-circulation of different strains during mumps outbreaks has been reported previously in Switzerland [Künkel et al., 1994; Ströhle et al., 1996] and more recently in China (personal communications with Dr. Li Wu). The evolution of the viruses during an epidemic is poorly understood and the accumulation of sufficient point mutations during virus transmission through multiple hosts in an epidemic to generate viruses of a

Fig. 4. Phylogenetic relationship between mumps viruses based on the SH gene sequences. All Portuguese sequences were generated during this study while the source of other sequences is reported elsewhere [Afzal et al., 1997].

different genetic lineage can not be ruled out totally. It will be of interest to monitor the development of further genetic diversity amongst mumps virus isolates in Portugal as well as in other European countries.

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